


REVIEW



The recycling regulation of sodium-hydrogen exchanger isoform 3 (NHE3) in epithelial cells

Ling Ran^a, Tao Yan^b, Yiling Zhang^a, Zheng Niu^a, Zifei Kan^a, and Zhenhui Song ^a

^aDepartment of Preventive Veterinary Medicine, College of Veterinary Medicine, Southwest University, Rongchang, China; ^bDepartment of Preventive Veterinary Medicine, College of Veterinary Medicine, Xinjiang Agricultural University, Urumqi, China

ABSTRACT

As the main exchanger of electroneutral NaCl absorption, sodium-hydrogen exchanger isoform 3 (NHE3) circulates in the epithelial brush border (BB) and intracellular compartments in a multi-protein complex. The size of the NHE3 complex changes during rapid regulation events. Recycling regulation of NHE3 in epithelial cells can be roughly divided into three stages. First, when stimulated by Ca²⁺, cGMP, and cAMP-dependent signaling pathways, NHE3 is converted from an immobile complex found at the apical microvilli (MV) into an easily internalized and mobile form that relocates to a compartment near the base of the MV. Second, NHE3 is internalized by clathrin and albumin-dependent pathways into cytoplasmic endosomal compartments, where the complex is reprocessed and reassembled. Finally, NHE3 is translocated from the recycling endosomes (REs) to the apex of epithelial cells, a process that can be stimulated by an increase in sodium-glucose cotransporter 1 (SGLT1) activity, epidermal growth factor receptor (EGFR) signaling, Ca²⁺ signaling, and binding to βPix and SH3 and multiple ankyrin repeat domains 2 (Shank2) proteins. This review describes the molecular steps and protein interactions involved in the recycling movement of NHE3 from the apex of epithelial cells, into vesicles, where it is reprocessed and reassembled, and returned to its original location on the plasma membrane, where it exerts its physiological function.

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Introduction

Sodium-hydrogen exchanger isoform 3 (NHE3, also known as SLC9A3), one of the nine subtypes of the Na⁺/H⁺ exchanger (NHE) in mammals, is part of the main pathway for the Na⁺ absorption and NaHCO₃ reabsorption in the intestinal and kidney brush border (BB) epithelial cells. NHE3 plays a key role in the absorption of salt and liquid, and pH stability, and is highly expressed in the apical membrane of the small intestine, colon and proximal tubules of the kidney [1,2]. Under physiological conditions, NHE3 exchanges intracellular H⁺ and extracellular Na⁺ at a 1:1 ratio of electrical neutrality for reverse transport and transmembrane exchange, maintaining the dynamic balance of intracellular pH¹. In addition, NHE3, is combined with a Cl⁻/base exchanger and a Cl⁻/formate or Cl⁻/oxalate exchanger to exert its salt reabsorption function [3], which accounts for

approximately 50% of the glomerular filtrate reabsorbed by NaCl and 70% reabsorbed by NaHCO₃ in the proximal tubule [4]. In addition to its role in Na⁺ and water absorption, NHE3 also regulates the absorption of other nutrients, such as dipeptides and amino acids, through H⁺-gradients [5].

Long term downregulation of NHE3 leads to a variety of pathophysiological conditions [6,7]. Research has shown that *Nhe3*^{-/-} mice had intestinal and kidney absorption defects, mild acidosis, and shortened lifespan [8]. In addition, the activity of NHE3 is closely related to the development of diarrhea [9–11]. Studies on NHE3 function and/or expression changes in patients with ulcerative colitis indicated that decreased NHE3 activity in the inflammatory mucosa might be the basis of ulcerative colitis diarrhea. A lack of NHE3 might be a key factor in immune regulation disorders, such as inflammatory bowel disease (IBD), loss of barrier

CONTACT Zhenhui Song  szh7678@126.com  Department of Preventive Veterinary Medicine, College of Veterinary Medicine, Southwest University, Rongchang, Chongqing 402460, China

Ling Ran and Tao Yan contributed equally to this work.

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function, and biological dysfunction, for example, in spontaneous colitis accompanied by mild diarrhea, occasional rectal prolapse, and weight loss in *Nhe3* KO mice [12]. Therefore, it is of great importance to determine the mechanism of NHE3 regulation associated with intestinal infection and inflammation, and water and salt absorption disorders in the gastrointestinal system and renal system.

The acute regulatory activity of brush border NHE3 contributes to the gastrointestinal and renal system water and Na⁺ homeostasis by changing the level of plasma membrane NHE3 via the rate of endocytosis and regulation of exocytosis [13]. Paradoxically, NHE3, which can perform the physiological function of sodium-hydrogen exchange, is fixed on the MV actin skeleton, with weak mobility. This fixation requires of NHE3 to combine with multiple PDZ domain scaffold protein Na⁺/H⁺ exchanger regulatory factors (NHERFs, including NHERF1, NHERF2, NHERF3, and NHERF4) and ezrin [14]. NHE3 is regulated by a large number of second messengers, including cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase II (cGKII), intracellular calcium ([Ca²⁺]_i), and protein kinase C alpha (PKCα) at the molecular and cellular level [15]. The regulation of NHE3 can occur through a variety of mechanisms, including vesicle endocytosis mediated by clathrin and albumin [16,17], intracellular protein transport, interactions with auxiliary regulatory proteins, and direct protein phosphorylation [18]. Although these regulatory processes are delicate and intricate, the recycling regulation of NHE3 is systematic, coherent, and complete. However, the importance and role of each molecular mechanism in regulating the activity of NHE3 has proven to be difficult to determine accurately. Therefore, this review provides a systematic description of the regulation of NHE3 activity, integrating the conclusions from on multiple previous studies related to the regulation of NHE3 activity.

NHE3 and its binding partners

NHE3 consists of two domains, a relatively conservative NH2 terminus (~450 aa) and a decentralized COOH terminus (~400 aa). The N-terminus

of NHE3 contains 13 transmembrane domains (Figure 1), which carries out the Na and H exchange. The C-terminus regulates the transport rate [1,19]. Almost all the rapid regulation of NHE3 activity requires the intracellular C-terminus (455–832 aa) [20]. Therefore, we discuss the C-terminus in detail in this review (Figure 1). The NHE3 C-terminus can be divided into four regions: (I) C-terminus domain 1 (F1 domain, 455–585 aa): There are two inosine phosphate binding regions (Tyr501–Arg512 and Arg520–Arg552) in the F1 domain, which are important for serum stimulation. Mutations in these regions alter the binding and basic NHE3 activity of phosphatidyl diphosphate PI (4,5) P2 and phosphatidyl triphosphate PI (3,4,5) P3. The latter alters the surface expression of NHE3, while the former does not [21]. In addition, there is a predicted helix structure between residues 511 and 528 [21,22]. On one side of the α helix, there is a cluster of three positive amino acids (K516, R520, and R527), including a conserved triserine cluster (Ser515, Ser522 and Ser526), which has a high isoelectric point (pI = 12.1). Point mutations of these positively charged amino acids eliminated Ezrin binding, which is a member of the Ezrin-radixin-moesin (ERM) protein family, connecting NHE3 to the cytoskeleton, and reduced the basic activity and surface of NHE3 [23]. The significant changes in phosphorylation of NHE3 at serine 552 and 605 were related to protein kinase A (PKA) activation, but did not directly affect the activity of NHE3 [18]. NHE3-Ser526 was predicted to be the phosphorylation site of serum/glucocorticoid-induced kinase 3 (GSK-3) [21,24]. (II) C-terminal domain 2 (F2 domain, 586–660 aa): Based on *in vitro* interactions, NHE3 binds NHERFs through internal PDZ domain binding motifs (589–660 aa) [25]. In mouse intestines and kidneys, cAMP acts by phosphorylating NHE3 primarily at two amino acids: Ser552 and Ser605 (in rabbit they are Ser554 and Ser607) [18,26,27]. Other consensus sequences for cAMP in NHE3 have been reported, including Ser330, Ser514, Ser576, Ser662, Ser691, Ser692, and Ser805; however, NHE3 does not require NHE3-ser663 phosphorylation [18,27]. The phosphorylation of Ser552 and Ser605 sites in NHE3 precede the inhibition of NHE3 activity by PKA [18], because mutation of Ser552 or Ser605 eliminated

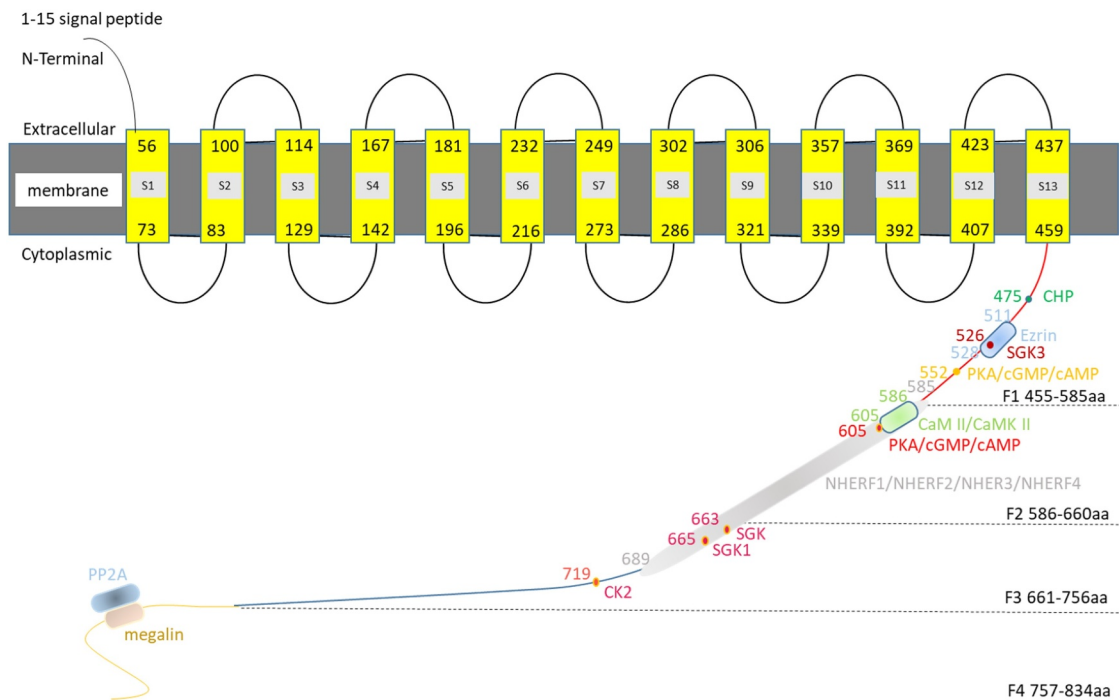


Figure 1. Simulation of NHE3 and its binding partners. Amino acids 1–477 of NHE3 (AAB48990.1) were subjected to prediction online using PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2/phyre2_output/6a92509e13ede827/summary.html). Amino acids 1–459 are the N-terminus NHE3, of which aa 1–15 form the signal peptide and aa 56–459 form the 13 transmembrane structure. Phyre2 predicted that 1–459aa belongs to the NH2 terminal of NHE3, in which 1–15aa was the signal peptide and 56–459aa was its 13 transmembrane structures. Y56-H73aa, P83-W100aa, V114-Y129aa, T142-I167aa, F181-V196aa, E216-E232aa, K249-S273aa, E286-E302aa, I306-K321aa, M339-S357aa, A369-I392aa, V407-V423aa and F437-I459aa belongs to S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13 transmembrane region, respectively. Its C-terminus can be divided into four domains: (a) The F1 domain (455–585 aa), in which aa 475 is the CHP binding site, and aa 526 is the SGK3 binding site. Amino acids 511–528 form the site where Ezrin binds directly to NHE3, and aa 552 is the PKA/cAMP/cGMP binding site; (b) The F2 domain 5 (86–660 aa): aa 586–605 are the CaM II/CaMK II binding site, and aa 605 is the PKA/cAMP/cGMP binding site; (c) The F3 domain (661–756 aa): aa 663 is the SGK binding site, aa 665 is the SGK1 binding site, and aa 719 is the CK2 binding site. The NHERFs (i.e. NHERF1, NHERF2, NHERF3, and NHERF4) binding sites spans the F2 and F3 domains (585–689 aa); (d) The F4 domain (757–834 aa), which contains the protein phosphatase 2A (PP2A) and megalin binding sites; however, their precise locations are unknown.

the inhibition of NHE3 by 8-Br-cAMP activated by PKA [18,28]. Calmodulin kinase II (CaMKII) is associated with the C-terminal 586–605 aa of NHE3 in a Ca^{2+} -dependent manner, and CaMKII requires 690 amino acids downstream of NHE3 to inhibit NHE3 phosphorylation and NHE3-related basic activities, while mutations in three recognized phosphorylation sites downstream of amino acid 690 prevented KN-93 (a CaMKII-inhibitor) from stimulating NHE3 activity [29]. (III) C-terminal domain 3 (F3 domain, 661–756 aa): Previously, residue S663 was proven to be necessary for acute stimulation of NHE3 by serum and glucocorticoid-inducible kinase (SGK) and glucocorticoid. Then, it was found that SGK1 could phosphorylate NHE3 at Ser665. Mutation of Ser665 blocked glucocorticoid regulation of NHE3. SGK1 phosphorylation regulates the activity of NHE3 by acidifying NHE3 via a

large phosphate side chain-induced allosteric displacement [26]. Tandem liquid chromatography (LC-MS/MS) of trypsinized NHE3 identified the phosphorylation site of casein kinase 2 (CK2) at the C-terminal S719 residue using an *in vitro* kinase assay. CK2 binds and phosphorylates the S719 site of the NHE3 C-terminus, and then stimulates the basic NHE3 activity of the C-terminus, thus affecting the transport of NHE3 [30]. (IV) C-terminal domain 4 (F4 domain, 757–832 aa): The interaction between NHE3 and megalin occurs in the MV gap, in which megalin-bound NHE3 is inactive [31]. By contrast, the association between NHE3 and dipeptidyl peptidase 4 (DPPIV) occurs mainly in the MV region, where NHE3 is active [31]. However, Kocisky et al. showed that there was a time separation between the phosphorylation and activity of NHE3, suggesting that

phosphorylation might not directly affect the transport activity of NHE3 [18].

The recycling regulation of hydrogen and sodium exchange of NHE3

Many membrane proteins, including transporters (such as NHE3), are involved in a recycling mechanism between intracellular compartments, including transgolgi, endosomes, and lysosomes in epithelial cells [32]. In fact, 70% of NHE3 subgroups seem to be tied to the cytoskeleton, resulting in limited movement and an inability to participate in endocytosis. Only those proteins that can diffuse into the pits covered by inclusion proteins can be internalized [33]. Generally, NHE3 recycles between the epithelial BB and intracellular compartments in a multi-protein complex, and the size of the complex changes with the rapid regulation of binding proteins, over a life span of about 16 h [22,34,35]. Recycling regulation of NHE3 in epithelial cells can be roughly divided into three stages: (I) When stimulated by Ca^{2+} , cGMP, and cAMP-dependent signaling pathways, NHE3 is converted from an immobile complex found at the apical MV into an easily internalized and mobile form that relocates to a compartment near the base of the intermicrovillar space; (II) NHE3 is internalized by clathrin and albumin-dependent pathways into cytoplasmic endosomal compartments, where the complex is reprocessed and reassembled [36]; (III) NHE3 is translocated from the RE back to the apex of the plasma membrane, a process that can be stimulated by an increase in SGLT1 activity, EGFR signaling, Ca^{2+} signaling, and the binding to β Pix (also known as ARHGEF7 (Rho guanine nucleotide exchange factor 7)) and SH3 and multiple ankyrin repeat domains 2 (Shank2) proteins, interacting with the actin cytoskeleton and retaining almost immobile subgroups on the plasma membrane in a manner dependent on the persistent activity of Rho GTPases.

Transfer of NHE3 from the apical membrane toward the intermicrovillar space

Normally, NHE3 is anchored on the actin scaffold, where it exchanges hydrogen and sodium with an immobile complex on the epithelial BB. Only when it transfers to the intermicrovillar space can

it be internalized. McDonough et al. have shown that NHE3 retracts from MV to the microvillar base in a two-step process in intact proximal tubules and then enters a denser membrane pool, which seems to be a reproducible storage pool [37,38]. Circulating cAMP, cGMP, elevated $[\text{Ca}^{2+}]_i$, as well as neurohumoral substances and bacterial toxins that cause increases in these second messengers, inhibit the activity of NHE3, thus triggering the transformation of NHE3 from an almost immobile complex retained on the apical membrane, in a manner dependent on the persistent activity of Rho GTPases, to an internalized and mobile NHE3 subgroup on the apical membrane, which stimulates NHE3 (Figure 2). Meanwhile, cAMP, and cGMP can also stimulate the secretion of Cl^- , increase the level of $[\text{Ca}^{2+}]_i$, and inhibit the activity of NHE3 [39]. Phosphorylated NHE3 forms membrane complexes and interacts with Ezrin, NHERFs, and actin to sense and maintain a constant direction during cell migration.

$[\text{Ca}^{2+}]_i$ regulates the phosphorylation of NHE3 activity, and the formation of multiprotein complexes

Carbachol (CCH) treatment of the intact rabbit ileum caused the rapid translocation of phospholipase C- γ (PLC- γ) to the apical membrane [40], resulting in activated PKC α moving to the apical plasma membrane to increase $[\text{Ca}^{2+}]_i$ through a muscarinic receptor (M3) in the basolateral membrane (BLM). This decreased the surface expression and activity of NHE3, and increased the volume of the NHE3 multiprotein complex [41], which inhibited NHE3 activity by 40%, and stimulated the endocytosis of NHE3. In addition, serotonin (5-HT), heat-stable *Escherichia coli* (STa) toxin B, and the rotavirus enterotoxin NSP5 directly upregulate $[\text{Ca}^{2+}]_i$, together with an ionophore. Previous studies have shown that $[\text{Ca}^{2+}]_i$ elevation in an NHERF2 (not-NHERF1)-dependent manner inhibits the activity of NHE3 at the apical plasma membrane [42]. NHERF2-NHE3 regulation involves the formation of polyprotein complexes (including NHE3, NHERF2, α -actinin-4, and PKC α). Thus, elevated $[\text{Ca}^{2+}]_i$ inhibits NHE3 activity by forming an oligomeric NHE3-

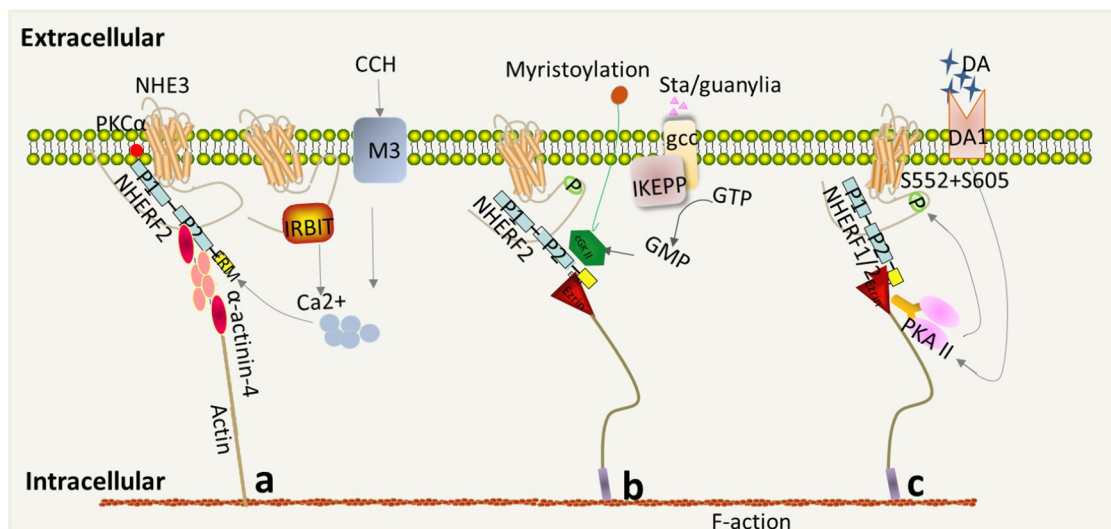


Figure 2. Transfer of NHE3 from the apical membrane toward the intermicrovillar space. (a) Elevated $[Ca^{2+}]_i$ inhibits NHE3 activity. Carbachol (CCH) raises $[Ca^{2+}]_i$ through the basolateral membrane (BLM) muscarinic receptor (M3), and the resulting activated protein kinase C alpha (PKC α) moves to the top of the plasma membrane. Elevated $[Ca^{2+}]_i$ oligomerizes through the formation of the NHE3-NHERF2-actin-4-PKC α complex, resulting in endocytosis of the plasma membrane-located NHE3, thereby inhibiting NHE3 activity; (b) Inhibition of NHE3 activity mediated by cGMP. Upon activation of guanylate cyclase, both guanylin and heat-stable enterotoxins (STa) bind to the same BB receptor, guanine cyclase-C, resulting in a rapid increase in intestinal cGMP levels. This activates BB cGKII, which passes its guanyl moiety N-terminally to the plasma membrane and the second PDZ domain of C-terminal NHERF2, to increase NHE3 phosphorylation at three sites (rabbit Ser554, Ser607, Ser663, equivalent to mouse Ser552, Ser605, Ser659), resulting in rapid inhibition of NHE3 activity; (c) cAMP-mediated inhibition of NHE3. Dopamine (DA), parathyroid hormone (PTH), vasoactive intestinal peptide, secretin, and enterotoxins, such as cholera toxin, activate adenylyl cyclase, which can rapidly increase the level of intestinal cAMP, resulting phosphorylation of NHE3 at Ser552 and Ser605, which then forms a complex containing NHE3, NHERF1/2, Ezrin, F-actin, and PKAII (cAMP-dependent protein kinase type II), thereby inhibiting the activity of NHE3.

NHERF2-actin-4-PKC α complex, resulting in endocytosis of NHE3 into the intermicrovillar space [42–44]. Interestingly, overexpression of α -actin-4 can enhance the inhibition of NHE3 activity by accelerating the oligomerization and endocytosis of NHE3. However, overexpression of the actin-binding domain plus spectrin repeat domain acted as a dominant negative mutation, which prevented the inhibition of NHE3 activity by a calcium ionophore and the oligomerization and endocytosis of NHE3 [42]. At the same time, elevated $[Ca^{2+}]_i$ decreases the intracellular NHE3-NHERF4 linkages to reduce NHE3-dependent NHERF4 targeting to the apex of the plasma membrane, and released NHERF3 from NHE3, resulting in decreased activity and surface expression of NHE3 [45]. It should be noted that the $[Ca^{2+}]_i$ -dependent inhibition mediated by NHE3 and NHERF2 has the opposite effect on the interaction between NHE3 and inositol 1,4,5-triphosphate (IP3) receptor-binding protein (IRBIT), which enhances the translocation of NHE3 to the surface membrane

through a mechanism involving calmodulin-dependent protein (CaM) and CaMKII [46].

Inhibition of NHE3 Activity Mediated by cGMP

After activation of guanylate cyclase, or the binding of guanylin and STa to the same BB receptor, guanosine cyclase-C, the intestinal cGMP level increases rapidly, and inhibits the absorption of NaCl by NHE3. The effect of cGMP on NHE3 seems to occur entirely through activation of cGKII in the BB [47]. The inhibition of NHE3 involves a complex, NHE3-NHERF2-cGKII/cGMP/cGKII, which increases the phosphorylation of NHE3 at three sites (rabbit Ser554, Ser607, Ser663, equivalent to mouse Ser552, Ser605, Ser659) and rapidly inhibits NHE3, resulting in a decrease in its surface expression. To inhibit NHE3, the N-terminus of myristoylated cGKII is attached to the plasma membrane, and its C-terminus is bound to NHERF2 (via PDZ domain 2) to allow cyclic GMP to inhibit NHE3, which effect involves the complex of NHE3-

NHERF2-cGKII [48]. Both cGMP and cGKII must be present simultaneously to enable cGMP to inhibit NHE3 [27]. Even if cGKII was co-expressed, it did not inhibit the stable transfection of NHE3 in PS120 cells. As Donowitz's research showed, most cell culture models (including PS120 cells and opossum kidney (OK) cells) lack cGKII, but have NHERF2, and thus cannot inhibit the activity of NHE3, showing that the presence of cGKII, rather than its absence, could inhibit NHE3 activity acutely. In addition, NHERF2 (but not NHERF1) PDZ2 C terminal binding to cGKII was identified under *in vitro* conditions. Although cGKII also binds NHERF2 *in vitro*, it will not cause NHE3 inhibition unless the myristoylation site is present, which is necessary for plasma membrane binding; the non-myristoylation mutant of cGKII does not support cGMP inhibition of NHE3 [49]. Guanylated cGKI can also reconstruct the inhibition of cGMP on NHE3, probably because NHERF2 is a specific cGMP kinase anchoring protein (GKAP) that binds to GKI and cGKII [48]. Neither cyclic cGKI nor cGKII lacking the myristoylation site could reconstitute cGMP inhibition of NHE3. In contrast, a chimera of wild-type cGKI and the first 29 amino acids of cGKII, which was myristoylated and thus bound to the plasma membrane, did reconstitute cGMP inhibition of NHE3. Thus, cGKII must be myristoylated and attached to the plasma membrane and bound to NHERF2 to inhibit NHE3 [49]. Therefore, cGMP's inhibition of NHE3 expression in fibroblasts and polarized epithelial cells requires both NHERF2 and cGKII. When NHERF2 was replaced by NHERF1, cGMP did not change the activity of NHE3 [49,50], indicating the strict requirement for NHERF2. The specific ligand of cGMP kinase also inhibits NHE3; therefore, a specific inhibitor of cGMP kinase blocked the effect of NHE3. In addition, because guanylate cyclase C binds to NHERF4, larger complexes in the BB might be involved in signal complexes associated with cGMP inhibition of NHE3.

cAMP-mediated inhibition of NHE3

Hormones (such as vasoactive intestinal peptide and secretin) and enterotoxins (such as cholera toxin) activate adenylate cyclase to rapidly increase

intestinal cAMP levels, which mediates the inhibition of NHE3 at Ser552 and Ser605 to decrease plasma membrane NHE3 levels, seemingly through another phosphorylation process involving PKA, which stimulates endocytosis [18]. Mutation of Ser605, the main target of PKA in NHE3, reduced the acute inhibition by cAMP by 50%[51]. In fibroblasts and polarized proximal tubular OK cell lines, cAMP inhibits NHE3 in the presence of NHERF1 or NHERF2, which involves the PDZ domain of NHERF1 or NHERF2 [52–54]. cAMP inhibition regulation includes the complex of NHE3, NHERF1 or NHERF2, Ezrin, F-actin, and protein kinase II [29,55]. Specifically, Ezrin located in F-actin binds to both NHERF1/NHERF2 and PKAII (cAMP-dependent protein kinase type II), positioning PKAII so it can phosphorylate NHE3, thus inhibiting its activity [55,56]. Therefore, the phosphorylation of NHE3 could directly inhibit its transport rate and also act as a signal for its endocytosis [3]. Kinetic analysis of NHE3 inhibition mediated by cAMP in OK cells showed that the number of transporters in the plasma membrane remained unchanged during the initial stage of cAMP-elevation [57], although a decrease was observed at subsequent time points [58].

In addition, Moe et al. found that parathyroid hormone (PTH) and Dopamine (DA) inhibited NHE3 activity in OK cells [59]. The short-term acute inhibition by PTH on NHE3 activity was not related to the change of abundance of NHE3 on the membrane; however, its long-term regulation of NHE3, which requires PKA-mediated C-terminal specific serine phosphorylation, decreased the amount of NHE3 [55]. DA significantly inhibits the activity of sodium and hydrogen exchange by reducing the surface abundance of NHE3, a process that also involves the phosphorylation of PKA-mediated transporters [58].

NHE3 enters the cytoplasm through internalization from the intermicrovillar space

Generally, NHE3 circulates between the plasma membrane and the intracellular compartments, which is regulated by changes in endocytosis and exocytosis rates or both to balance the amount of NHE3 on the surface of the plasma membrane

[60,61]. Most basic NHE3 endocytosis occurs through clathrin and albumin-dependent processes, partially involving lipid rafts (e.g. CCH-mediated Ca^{2+} dependent NHE3 internalization) [34,62] (Figure 3).

Endocytosis of NHE3 via clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the most distinctive endocytosis mechanism, and is the main way for epithelial cells to absorb macromolecules [63,64]. NHE3 is internalized in a manner similar to the multiple receptors of receptor-mediated endocytosis via clathrin-mediated pits and vesicles, which are then transported to early endosomes (EEs) through endocytotic vesicles, involving a coatamer protein complex subunit epsilon (ϵ -COP)-dependent step [61,63]. The internalization pathway of the NHE3-clathrin-dependent complex mainly involves the following proteins: (I) Synaptotagmin I

(Syt I), a Ca^{2+} binding protein, which is expressed in the apex and subapical areas of the intestinal epithelial cells, with its highest expression in villous epithelial cells and the surface of crypt epithelial cells in the intestine. Syt I is a pivotal mediator of Ca^{2+} and cAMP-induced membrane endocytosis of NHE3, and is a member of the large class of Syt proteins that are involved in synaptic vesicle fusion as well as the delivery of vesicles from the Golgi to the synaptic bulb [65]. The endocytosis process of NHE3 by Syt I involves many different transport proteins, such as vesicle-associated membrane proteins, synaptic fusion proteins (syntaxin), S-nitroso-N-acetyl penicillamine (SNAP), and adaptor protein 2 (AP2) [66,67]. Mechanistically, Syt I binds to intestinal epithelial NHE3 and activates the assembly of clathrin required for endocytosis through the recruitment of AP2 and clathrin [65], mediating the endocytosis induced by cAMP and Ca^{2+} [58,65]. It should be noted that NHERF1 mediates cAMP

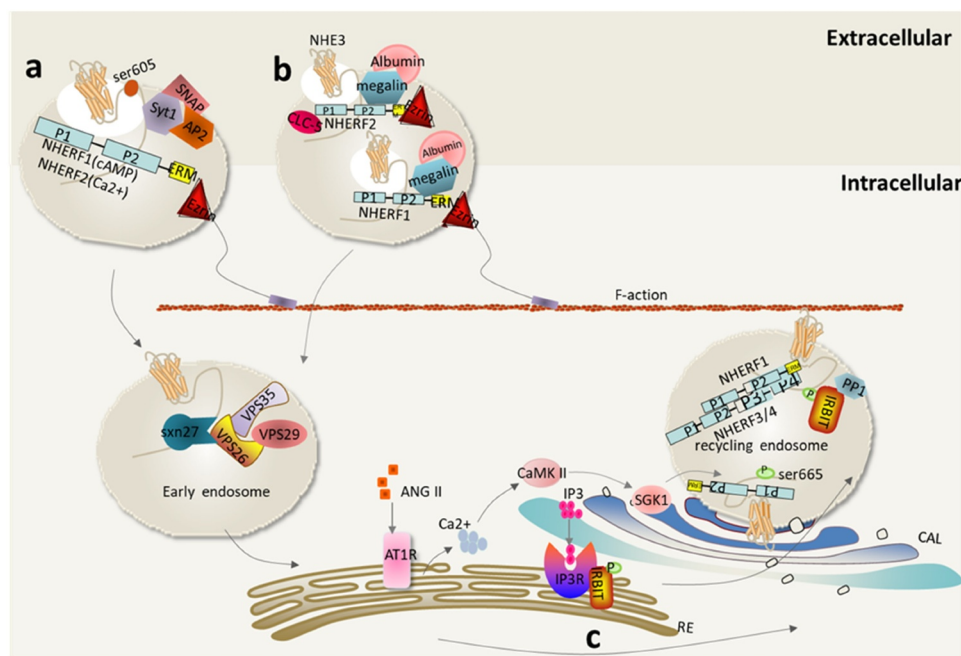


Figure 3. NHE3 enters the cytoplasm through internalization from the intermicrovillar space. (a) Endocytosis of NHE3 mediated by clathrin (CME). Synaptotagmin I (Syt I) binds to NHE3 in the intestinal epithelium and initiates the assembly of clathrin required for endocytosis through the recruitment of AP2 and clathrin, mediating endocytosis induced by cAMP (mediated by NHERF1) and Ca^{2+} (mediated by NHERF2). In addition, DA phosphorylates NHE3 (Ser605) mediated by PKA, leading to NHE3 endocytosis through reticulon-mediated vesicles; (b) Albumin mediated NHE3 endocytosis. Albumin-dependent NHE3 endocytosis requires the scavenger receptor megalin to bind to albumin in the intravillar cleft to form a large protein complex, which is then regulated by many protein complexes of transmembrane proteins and auxiliary proteins (e.g. scaffold proteins NHERF1 and NHERF2). The type 2 PDZ binding domain of NHERF1 binds directly to C-terminus of megalin and NHE3. NHERF2 nucleates endocytotic complexes in villi by interacting with NHE3 and CLC-5; (c) Reprocessing, assembly, and cycling of NHE3 in the cytoplasm. The PDZ domain of SNX27 binds VPS26 of the retromer complex (including VPS26-VPS29-VPS35) and regulates NHE3 exocytosis from early endosomes (EEs) to the plasma membrane.

regulation [68], while NHERF2 mediates Ca^{2+} regulation of NHE3 [42]. The binding and internalization of NHE3 serine 605 (S605) and Syt I were identified using fluorescence resonance energy transfer (FRET) analysis and NHE3 site directed mutation. (II) DA, a key hormone for sodium balance in mammals, significantly reduces the amount of surface NHE3 and inhibits the exchange activity of Na^+/H^+ at the apex of proximal tubules [69], mainly through PKA-dependent and PKA-independent mechanisms involving DR1-like receptors, one of the molecular subtypes of the DA receptor, which results in NHE3 endocytosis via clathrin-coated vesicles [58,70]. In addition, studies have shown that DA-stimulated NHE3 endocytosis can be blocked by drugs, genetic inhibition of PKA, or mutation of two PKA-binding residues (Ser-560 and Ser-613) on NHE3. Another important process of endocytosis mediated by clathrin in the early stage is the pH homeostasis and intracellular fusion events in the endosomal compartment, which might affect ligand-receptor separation, vesicle transport, endosomal fusion events, recycling to the plasma membrane, and the formation of outer membrane proteins. However, pH homeostasis is partly maintained by vacuolar H^+ -ATPase and NHE3 [71].

NHE3-dependent albumin endocytosis

NHE3-dependent albumin endocytosis requires macrophage scavenger receptors to bind to albumin in the intravillar cleft to form a large protein complex containing many transmembrane proteins and accessory proteins. This complex may include accessory proteins, such as the type I PDZ motif (S/T-X- Φ) of the scaffold proteins NHERF1 and NHERF2, which is then subjected to endocytosis [17,62,72]. Previous studies have shown that the interaction between NHERF scaffolds and members of the albumin endocytosis complex is as follows: Firstly, megalin is important in regulating albumin uptake, especially its ability to translocate albumin into Villar fissures [73]; Secondly, the PDZ binding motif of megalin at its C-terminus has been proven to bind proteins containing MAGI-1 and postsynaptic density (PSD)-95 PDZ domains [74], such that the effect of NHERF1 may be achieved through a direct link with scavenger receptors. Fortunately, Slattery et

al. found that NHERF1 directly bound to NHE3 and the C-terminus of megalin through the type 2 PDZ binding domain of NHERF1, without any auxiliary proteins, i.e. NHE3 was bound to this region [17]. In contrast, NHERF2 nucleates endocytic complexes in the villi through an interaction with NHE3 and chloride voltage-gated channel 5 (CLC-5), to stimulate the endocytosis of NHE3 [73]. Silencing *NHERF1* increased the endocytosis of albumin, probably because the absence of NHERF1 increased the mobility of NHE3 in the MV, thereby increasing the formation of endocytosis complexes in the villous fissure. Silencing *NHERF2* reduced albumin endocytosis [62], presumably by increasing and reducing CLC-5 cell surface availability, respectively.

Endocytosis mediated by cell division cycle 42 involves lipid rafts independent of clathrin

Nearly half of surface NHE3 is located in lipid rafts, and the activity and transport of NHE3 depends on these lipid rafts [60], which might be very important for the timing of membrane signaling, transport, and transport activities of NHE3. It is worth noting that the endocytosis of NHE3 mediated by CCH occurs via lipid rafts, activated by the cell division cycle 42 (Cdc42)-dependent pathway, which does not involve clathrin. Cdc42 is necessary for a clathrin-independent endocytic process, termed the clathrin Independent Carrier (CLIC)/GPI anchored protein-enriched Early Endocytic Compartment (GEEC) pathway [75,76]. Research shows that NHE3 binds to NHERF3 in the plasma membrane, and is then internalized to a compartment near the base of the MV after CCH treatment, while NHERF3 remains in the plasma membrane [77].

Reprocessing, assembly, and cycling of NHE3 in the cytoplasm

By comparing the size of the surface and endosomal compartment-located NHE3, it was found that the NHE3 complex in the plasma membrane was larger than that in the cell, suggesting that not all the complexes are formed in the ER or GOLGI, and it could be assumed that NHE3 is associated with several different proteins as it passes between the BB and the recovered endosome. Therefore, the

complex containing NHE3 is dynamic (Figure 3). During the cyclic processing of receptors and ligands, internalized receptors and ligands either enter REs for reprocessing or are transported into late endosomes and then degraded by lysosomes. An important process of the endocytosis pathway is acidification of the endosomal compartments [64,78].

SNX27 is necessary for the transfer of NHE3 from early endosomes to the extracellular region

Sorting nexin 27 (SNX27), a member of the sorting nexin family, contains a PDZ domain that has high homology with the sequence of the NHERF family's PDZ domain, which is necessary for the transfer of NHE3 from the EE to extracellular region of epithelial cells. The multimeric complex of NHE3 regulates its activity by interacting with PDZ domain proteins associated with NHERF proteins [20,79]; therefore, it is possible that other proteins also interact with the SNX27-NHE3 complex to regulate the transport of NHE3 *in vivo*. NHE3 mainly interacts with the SNX27 N-terminal PDZ domain, which lies upstream of the PX domain, unique in SNX27 [80,81]. In addition, SNX27 is annotated as having a Ras-association domain and an extended C-terminal domain [82,83]. SNX27 was initially thought to mediate intracellular transport to late endosomes leading to degradation [84,85]. Then, later research suggested that SNX27 is more likely to mediate the transport of Rab5 to Rab11-positive vesicles, while it remains in Rab5-positive EE vesicles [86]. Singh et al. have revealed the mechanism of SNX27's regulation of NHE3: SNX27 binds to and regulates the exocytosis of NHE3 from EEs to plasma membranes, and plays an important role in regulating the transport of multiple protein membranes in the EEs, which occurs through interaction with a retromer complex [35]. The retromer complex comprises a vacuolar protein sorting (VPS) trimer core subcomplex (VPS26-VPS29-VPS35) and the membrane-related sorting nexin (SNX) dimer (SNX1-SNX2) [87]. In this process, SNX27 binds VPS26 directly via SNX27's PDZ domain, which enhances serine phosphorylation of the SNX27 PDZ domain, inhibits the binding of SNX27 to NHE3, and reduces the extracellular

secretion of NHE3 to the plasma membrane. In summary, the PDZ adapter protein SNX27 and other nucleosomes may appear along the endosomal-recycling pathway [88]. In addition, the retention of NHE3 on plasma membrane requires SNX27 regulation, and deletion of SNX27 reduced the apical localization and basic activity of NHE3 in intestinal epithelial cells. On the other hand, NHE3 and NHERF3 and/or NHERF4 were colocalized in Rab11 positive RE vesicles, and NHE3-NHERF3 translocated from this chamber to the plasma membrane after the Ca^{2+} concentration increased, while NHERF4 remained in the Rab11 vesicles, indicating the motivational role of Rab11-positive REs in the regulation of NHE3 [45,77,88].

Transcriptional regulation of NHE3: glucocorticoid/ SGK1

Most NHE3 regulation is the acute regulation that occurs within a few minutes to a few hours after activation, which is rapid and reversible, often involving changes in phosphorylation, transport, and dynamic interactions with regulatory proteins. By contrast, the chronic regulation of NHE3 involves the transcription and translation modification of NHE3. In short, the regulation of NHE3 is complex, with myriad signals converging on a single protein at different levels. A major example of the complex and integrated regulation of NHE3 is the regulation of glucocorticoids, which increase the level of *NHE3* mRNA and often affect NHE3 via multiple pathways, a process that requires SGK1 as a key kinase targeting the S663 site of NHE3 [89–92]. *In vitro*, after activation by serum glucocorticoids, SGK1 induced the translation activity of NHE3 requiring NHERF2 (not NHERF1) [93], and NHERF2 interacts with actin via ezrin [94], causing the translocation of NHE3 to the cell surface [91]. These findings are supported in mice lacking SGK1 expression: Glucocorticoid-mediated stimulation activity and translocation of NHE3 to the apical membrane of mice was significantly reduced [95]. The upregulation of NHE3 function mediated by SGK1 also includes phosphatidylinositol 3 (PI3) kinase, which is known to affect the surface expression of NHE3 [92].

NHE3 translocation from recycling endosomes (REs) to the apex of epithelial cells

There are several types of apical membranes containing NHE3 in epithelial cells, including NHE3 in the lipid raft and outside the lipid raft on the plasma membrane. NHE3 binds to megalin (denser, less active) but not to megalin (lighter, more active). Intracellular NHE3 also exists in two pools, one of which is rapidly discharged, while the other moves to the apical membrane with slower kinetics [33,36]. When stimulated by certain signals, such as increased SGLT1 activity, EGFR signaling, elevated Ca^{2+} concentration, and binding to β Pix & Shank2 proteins, NHE3 will be translocated from the REs back to the apex of the plasma membrane (Figure 4).

The SGLT1/P38AMP/MAPKAPK2/PI3K/AKT2/Ezrin pathway activates NHE3

Glucose transport triggered by SGLT1 will lead to the activation of the apical hydrogen-sodium exchanger NHE3 [96]: Na^+ -glucose co-transport mediated by SGLT1 activates phosphatidylinositol 3-kinase (PI3K), Serine/Threonine Kinase 2 (AKT2) and NHERF2-dependent signaling pathways via p38 mitogen-activated protein kinase (p38AMPK)/MAP kinase-activated protein kinase 2 (MAPKAPK2) sequentially to stimulate NHE3 activity, and finally AKT2 phosphorylates Ezrin [2]. PI3K is a cytoplasmic signaling molecule that is recruited to the activated receptor under stimulation, which leads to an increase in the intracellular 3-phosphoinositide phospholipid level and induces a signal response, including AKT2 activation [94]. AKT2

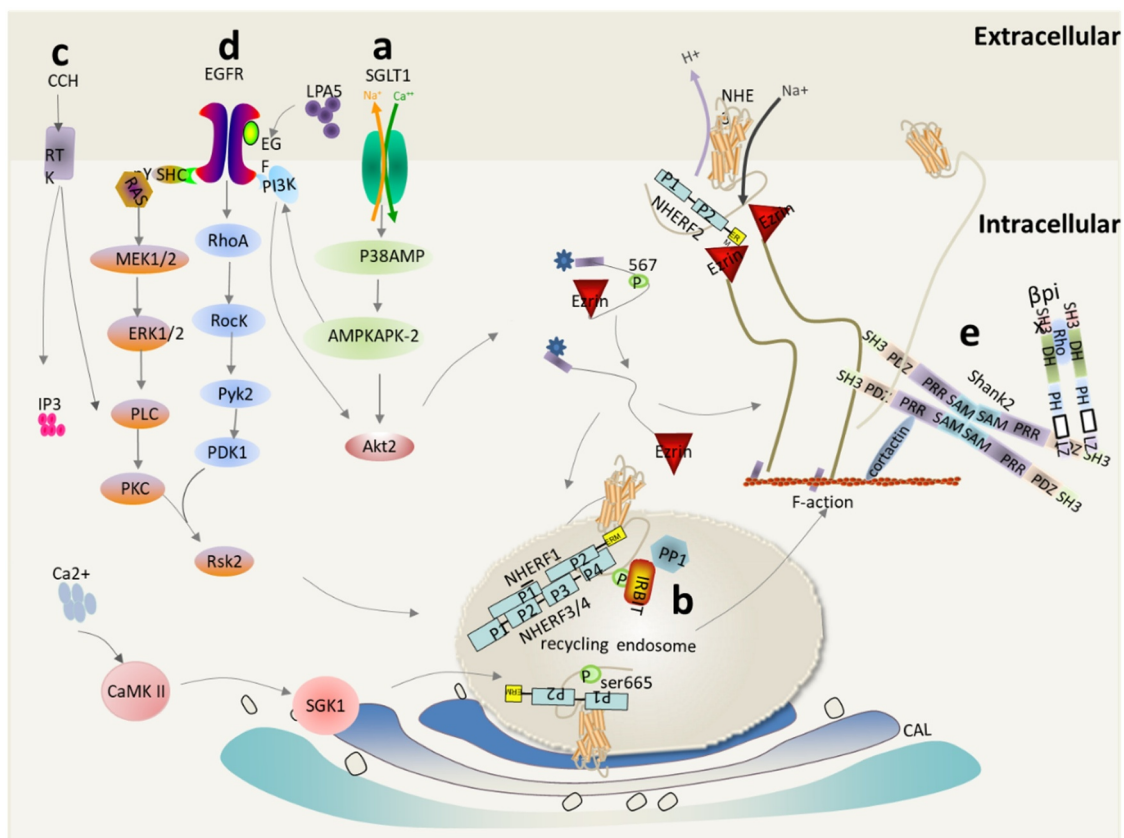


Figure 4. NHE3 translocation from REs to the apical region of epithelial cells. This mainly includes the following activation mechanisms: (a) The SGLT1/P38AMP/AMPKAPK-2/PI3K/AKT2/Ezrin pathway activates NHE3; (b) IRBIT mediates the activation of NHE3 by ANG II via a Ca^{2+} /calmodulin-dependent protein kinase II-dependent pathway; (c) Regulation of NHE3 by PLCs; (d) Regulation of NHE3 by the (2AR)/LPA5/EGFR/RSK2 pathway; (e) β Pix upregulates NHE3 through protein-protein interaction mediated by Shank2.

phosphorylates Ezrin to trigger the translocation and activation of NHE3 after the initiation of Na-glucose transport [97]. Ezrin connects with the actin cytoskeleton in MVs and is necessary to regulate the rate of endocytosis of NHE3 and changes to stimulated extracellular proliferation [97]. In addition to directly binding to NHE3, Ezrin interacts with the regulatory subunit p85 of PI3K at two different sites, one is the N-terminal amino acid 1–309, and the other is its C-terminal phosphorylated tyr353. Binding of residues to the SH2 [98]. In addition, Ezrin regulates NHE3 activation through a Rho-dependent mechanism. The Rho-dependent formation of an MV-like structure requires the ERM protein (standing for Ezrin, radixin, and moesin), which is controlled by the small GTPase Rho and phosphorylated by RhoK on a C-terminal threonine to interfere with their head-tail interaction [98]. Therefore, the lateral migration of NHE3 regulated by Ezrin activation on the apical plasma membrane is Rho/RhoA- RhoA-Rho associated kinase (ROCK)-dependent (not shown in the picture) rather than PI3K/AKT2-dependent [23]. In addition, the N-terminal region binding sites between Rho-GDI and NHERF1 proteins have been identified [99].

IRBIT/PLCs mediate the activation of NHE3 by ANG II via a CaMKII-dependent pathway

The interaction between NHE3 and inositol 1,4,5-triphosphate (IP3) receptor binding protein (IRBIT) is opposite to the $[Ca^{2+}]_i$ -dependent inhibition mediated by the combination of NHE3 and NHERF2. IRBIT, dependent on CaM and CaMK II, mediates the activation of NHE3 by ANG II and enhances the translocation of NHE3 to the surface membrane [46]. IRBIT binds to the carboxyl terminal region of NHE3, which is necessary for the acute regulation of NHE3, responding to the increase in Ca^{2+} induced by thapsigargin or ionomycin in PS120 fibroblasts and activating NHE3 [46]. In addition, extracellular stimulation, including hormones, growth factors, and neurotransmitters activate receptor tyrosine kinase (RTK), and then activate PLCs (PLC β and PLC γ) through their phospholipase catalytic domains. The activated PLCs hydrolyze the membrane and bind phosphatidylinositol 4,5-bisphosphate (PIP2) to produce two second messengers: Diacylglycerol

(DAG) and IP3. The former is an activator of PKCs, and the activated PKC α translocates to the BB [100]; while the IP3 binds to the IP3 receptor (IP3R) to induce a transient increase in Ca^{2+} through its release from storage in the endoplasmic reticulum [101–104]. At the same time, the PLC γ lipase-dependent increased in Ca^{2+} also activates the calmodulin/calmodulin kinase II (CaM/CaMKII) complex.

CaMKII is a multifunctional kinase that is involved in many biological processes regulated by $[Ca^{2+}]_i$, including synaptic transmission, gene transcription, and channel regulation [105]. Reversal of phosphorylation indicated that NHE3 was phosphorylated by CaMKII under basic conditions, suggesting that CaMKII is a novel NHE3 binding protein, and that this association was reduced by $[Ca^{2+}]_i$ elevation, which occurs in fibroblasts and the BB [106]. The angiotensin II (ANG II) system, which is mainly involved in regulating blood pressure and fluid balance, has been confirmed to stimulate the activity of NHE3 in renal proximal tubular epithelial cells [107]. The regulation of NHE3 activity by ANG II is mediated by activation of the G protein-dependent angiotensin II type I receptor (AT1R)/PLC/calcium/calmodulin pathway: ANG II activates and phosphorylates CaMKII directly or indirectly through AT1R and the CaM complex, inducing increased $[Ca^{2+}]_i$ to stimulate NHE3 activity. Ang II (1 nM) did not change the total expression of NHE3, but induced CaMKII to stimulate NHE3 phosphorylation and CD40 receptor associated factor 1 (CRAF-1)/mitogen-activated protein kinase (MEK)/ERK1/2 to stimulate 90 kDa ribosomal protein S6 kinase 1 (p90RSK) activity [108]. In addition, ANG II stimulates NHE3 activity by increasing the extracellular insertion of NHE3 in a phosphoinositide 3-kinase (PI3K)-dependent manner [109]. Recently, Peijian et al. found that ANG II induced an increased interaction among NHERF1, NHE3, and IRBIT, and they were co-expressed in the same macro-complex. NHERF1 was essential for the positive RE transport and activation of ANG II-mediated NHE3 in cultured cells. When the NHERF1 PDZ1 domain was removed, the interaction between NHERF1 and IRBIT was eliminated. In addition, the phosphorylation of IRBIT at Ser68 is necessary for the

assembly of the NHEF1-IRBIT-NHE3 complex [110].

Regulation of NHE3 by the LPA5/EGFR/RSK2 Pathway

Lysophosphatidic acid (LPA), a small phospholipid that is produced by various kinds of cells or derived from food, stimulates NHE3 activity via lysophosphatidic acid receptor 5 (LPA5) transactivation of epidermal growth factor receptor (EGFR) in the apical membrane [111,112]. The activation of NHE3 by the LPA5/EGFR/RSK2 pathway occurs as follows: Transactivation of EGFR activates the proline kinase 2 (PYK2)-RhoA-RocK pathway and the downstream MEK-ERK pathway, and the two pathways eventually converge on the central protein RSK2 (also known as p90RSK). Then, LPA5 phosphorylates RSK2 at T573 and S363 via ERK, and then RSK2 self-phosphorylates at S380. At the same time, RhoA and RocK are involved in the activation of PYK2, which phosphorylates and activates pyruvate dehydrogenase kinase 1 (PDK1) bound to S380 and S221-phosphorylated RSK2. Finally, fully activated RSK2 directly interacts with NHE3 and phosphorylates it at S663. In conclusion, these results reveal the key role of EGFR in LPA's regulation of NHE3, and indicate that the RhoA-RocK-PYK2 and MEK-ERK pathways converge on NHE3 [113]. RSK2 is a member of the Ser/Thr protein kinase P90 ribosomal S6 kinase (RSK) family, which responds to certain growth factors and mitogens, including epidermal growth factor, insulin, and insulin-like growth factor-1 [114]. PYK2, as a pH sensor to initiate an acid regulation cascade in NHE3 regulation, which acts as a scaffold for SRC-phosphorylated PDK1, is necessary for c-SRC kinase and NHE3 acid activation [115]. In addition, PI3K is necessary for EGF/fibroblast growth factor (FGF) stimulation of NHE3 in fibroblasts and EGF in the ileal BB to stimulate NHE3.

β Pix up-regulates NHE3 through protein-protein interaction mediated by Shank2

β Pix, a small GTPase in the Rho family of guanine nucleotide exchange factors (GEFs), is a strong binding partner of Shank2 [116]. Shank2 is a newly identified scaffold protein and is considered as the central coordinating protein of the

postsynaptic density (PSD) membrane protein complex [117], containing many specific protein-protein interaction sites, including an anchor protein repeat sequence, an SH3 domain, a PDZ domain, a proline-rich region, and a sterile motif (SAM) [118]. Shank interacts with β Pix to promote the accumulation of β Pix-related signal molecules in the synapses of excitatory postsynaptic density membrane, regulates the membrane expression and activity of NHE3 by activating ρ GTPase, small GTPases of the Rho family, and Shank2 protein in the apical region of epithelial cells [119,120]. In addition, the interaction between the PDZ domain of the Shank family and RSK2 (also containing a PDZ binding motif at its C-terminus), has been proven to regulate synaptic transmission through the amino-3-hydroxy-5-methyl-4-isoxazopropionic acid receptor [121]. Thus, it is likely that RSK2-mediated phosphorylation of NHE3 might enhance its interaction with other proteins, such as Shank2 and Ezrin, which can link to NHE3 in the apical membrane, because NHERF2 itself is not sufficient to address the specificity of RSK2 [122]. In addition, in Caco-2 epithelial cells, decreased Shank2 expression by RNA interference led to decreased expression and activity of NHE3 and enhanced the inhibition of NHE3 by cAMP [120]. These results suggested that Shank2 is a novel NHE3 interacting protein that participates in the fine regulation of transepithelial salt and water transport by affecting the expression and activity of NHE3.

Conclusions and Perspectives

NHE3, as an important exchanger of Na^+/H^+ in the gastrointestinal tract, does not exist in the form of monomers, but in the form of complex and dynamic macromolecular complexes. The complexes, mainly including the PDZ domain proteins (NHERF1-4) of the NHERF family and the ligand protein Ezrin, are involved in phosphorylation, transport of multiple proteins, and multiple cellular pathways that interact with cellular proteins, which are integrated to regulate NHE3 activity. Acute regulation of NHE3 activity is achieved by redistribution of NHE3 between the plasma membrane and the intracellular compartments. In this review, we presented the regulatory mechanism of NHE3 (Figure 5), i.e. (1) Normally,

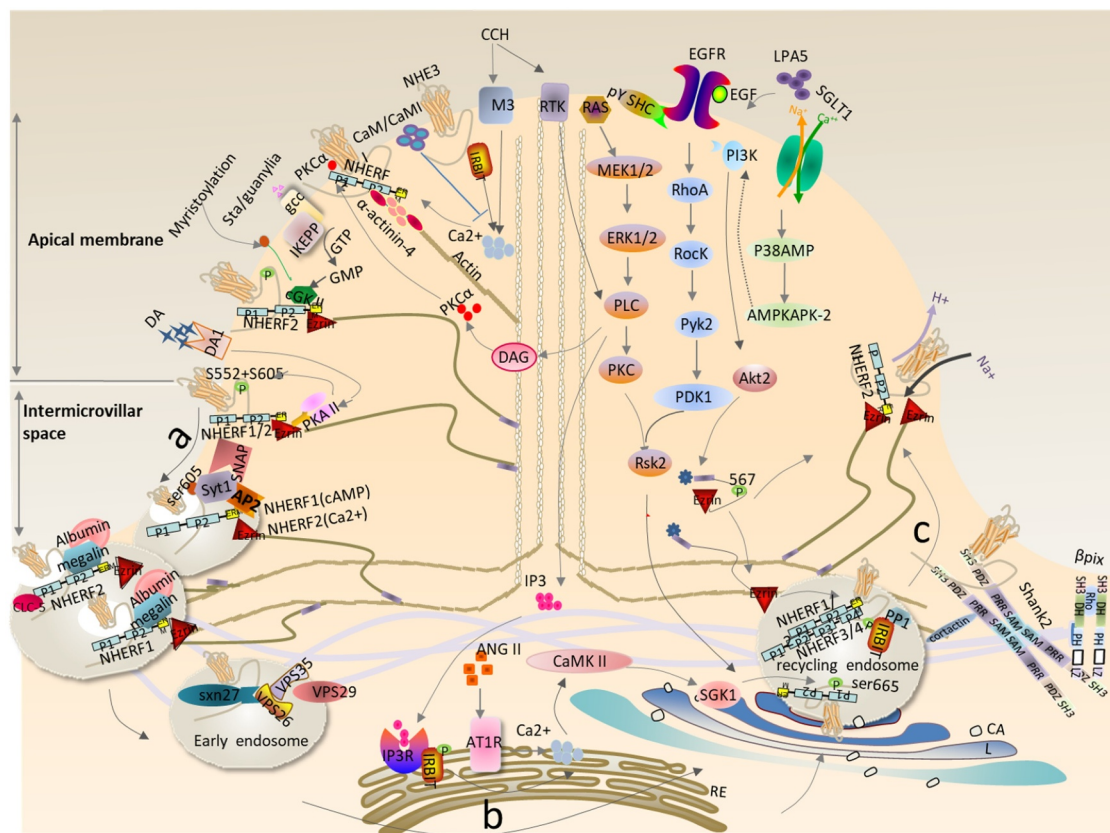


Figure 5. Cyclic physiological regulation of NHE3 for sodium and hydrogen exchange in epithelial cells. (a) Transfer of NHE3 from the apical region of epithelial cells to the interstitial space. Under normal physiological conditions, NHE3 circulates between the plasma membrane and intracellular compartments. In fact, about 70% of the NHE3 subgroups seem to be tied to the cytoskeleton and play a stable role in the exchange of hydrogen and sodium. When stimulated by plasma membrane Ca^{2+} /cAMP/cGMP and other signals, the NHE3-binding complex undergoes a series of complex dynamic changes, which transforms the stable NHE3 subgroups into movable subgroups; (b) NHE3 enters the cytoplasm through internalization from the intermicrovillar space. The movable NHE3 subgroups transfer to the intestinal intermicrovilli space. Only those NHE3 subgroups transferring to the intermicrovilli space can enter the inclusion protein through such pathways as clathrin/albumin/lipid rafts. Encapsulated vesicles can then be internalized. NHERF1 mediates cAMP regulation of NHE3, while NHERF2 mediates Ca^{2+} regulation of NHE3. Subsequently, internalized NHE3 is reprocessed, assembled, and recycled in the cytoplasm and assembled into circulating nucleosomes via SNX27; (c) NHE3 translocation from the RE to the apical region of the epithelial cells. When NHE3 in circulating nucleosomes is stimulated by plasma membrane activation signals, such as Ca^{2+} /Ezrin/RSK2/Shank2, it will migrate to the plasma membrane and be re-fixed on the cytoskeleton to form stable subgroups of NHE3, thus exerting the physiological activity of hydrogen and sodium exchange.

when stimulated by Ca^{2+} , cGMP, and cAMP-dependent signaling pathways, NHE3 binds to the cytoskeleton in an immobile complex state to participate in hydrogen-sodium exchange at the apical MV, and is then converted from the immobile complex into an easily internalized and mobile form that relocates to a compartment near the base of the MV; (2) Subsequently, it is endocytosed into intracellular vesicles mainly via clathrin/albumin mediation pathways, passing through the Rab5-positive early endosomes (EE) and reorganized/reprocessed in the Golgi and endoplasmic reticulum,

before being transferred to the Rab11a- positive RE; (3) NHE3 is translocated from the RE back to the apex of the plasma membrane, a process that can be stimulated by the SGLT1/P38AMP/MAPKAPK2/PI3K/AKT2/Ezrin pathway, IRBIT/PLCs mediating ANG II via a CaMKII-dependent pathway, LPA5/EGFR/RSK2 signaling, and by binding to β Pix & Shank2 proteins. Our understanding of the relationship between water and salt absorption disorder in the gastrointestinal system and renal system and NHE3 activity, the protective mechanism of NHE3 on diarrhea, and the mechanism of microbial or

inflammatory stimuli inhibiting NHE3 that leads to diarrhea remains limited; therefore, the complex dynamic changes and synergistic properties of NHE3-interacting proteins should be the subject of future research. It is of great practical significance to study the regulation mechanism of NHE3 activity for the treatment of diarrhea, intestinal infection and inflammation, and water and salt absorption disorders in the gastrointestinal system and renal system in the future.

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ORCID

Zhenhui Song  <http://orcid.org/0000-0002-2558-3164>

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